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## Decreased Lectin-Like Oxidized LDL Receptor 1 (LOX-1) and Low Nrf2 Activation in Placenta Are Involved in Preeclampsia

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**Context:** Serum concentration of oxidized low-density lipoprotein (oxLDL) is higher in women with preeclampsia than in normal pregnant woman. Lectin-like oxLDL receptor-1 (LOX-1) is one of the scavenger receptors for oxLDL and is abundantly expressed in placenta. It is well known that oxLDL activates nuclear factor erythroid 2-related factor 2 (Nrf2), a master regulator of antioxidant and cytoprotective genes such as heme oxygenase-1 (HO-1), which play an important role in preeclampsia. However, it has yet to be elucidated whether LOX-1, along with Nrf2, participates in the pathology of preeclampsia.

**Objective:** The objective of the study was to assess LOX-1 expression and Nrf2 activation in pre-eclamptic placentas and to manifest their physiological roles in preeclampsia.

**Methods:** Expression and regulation of LOX-1, HO-1, and Nrf2 were evaluated by real-time quantitative RT-PCR and Western blotting. The functions of LOX-1 and Nrf2 were examined using an anti-LOX-1 antibody and Nrf2 activator in JAR, a choriocarcinoma cell line, and placental explants.

**Results:** Both LOX-1 expression and Nrf2 activation were significantly decreased in preeclamptic placentas compared with normal controls. A significant decrease in LOX-1 mRNA was found in placental explant cultures under hypoxic conditions. Activation of Nrf2 up-regulated HO-1 in both the JAR cells and placental explants. Furthermore, oxLDL increased HO-1 mRNA, whereas the blockade of LOX-1 inhibited the increase of HO-1 mRNA in JAR cells.

**Conclusion:** Decreasing LOX-1 expression in preeclamptic placenta may contribute to high oxLDL concentration, low Nrf2 activation, and low HO-1 expression. These findings provide novel insights into the crucial role of LOX-1 and Nrf2 in the pathogenesis of preeclampsia. (*J Clin Endocrinol Metab* 97: E1862–E1870, 2012)

Preeclampsia is a pregnancy-specific multisystem disorder, generally defined as new hypertension and substantial proteinuria at or after 20 wk of gestation (1, 2). Despite the numerous clinical and basic studies, its etiology and pathogenesis remain enigmatic, and only delivery is regarded as a fundamental resolution. Therefore, preeclampsia is still a leading cause of maternal and perinatal mortalities and morbidities.

Maternal hyperlipidemia is one of the striking changes to take place in lipid metabolism during even normal preg-

nancy (3). In women with preeclampsia, serum levels of triglycerides, low-density lipoproteins (LDL) are higher than those in normal pregnant women (4). In addition, accumulating reports suggest that serum oxidized LDL (oxLDL) are higher in preeclampsia (5, 6). OxLDL is essential in the genesis and progression of atherosclerosis (7). It can lead to endothelial dysfunction, a key feature of preeclampsia (8), by binding to its scavenger receptors including lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1), CD36 and scavenger receptor A (SR-

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Abbreviations: DEM, Diethylmaleate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HO-1, heme oxygenase-1; LDL, low-density lipoprotein; LOX-1, lectin-like oxLDL receptor-1; nLDL, native LDL; Nrf2, nuclear factor erythroid 2-related factor 2; oxLDL, oxidized LDL; sFlt-1, soluble fms-like tyrosine kinase 1; SR-A, scavenger receptor A.

A). LOX-1 is a 52-kDa, type 2, single-transmembrane receptor cloned by Sawamura in 1997 (9) and present primarily on endothelial cells and macrophages (10). Although the basal expression of LOX-1 is very low, it can be rapidly induced by various stimuli and several pathological conditions, such as oxLDL, diabetes (11), and hyperlipidemia (12). Intriguingly, placenta is the organ with the highest LOX-1 expression, even in a healthy state (9), suggesting that LOX-1 is crucial for maintaining pregnancy. However, the underlying precise mechanism by which LOX-1 elicits its effects in placenta and contributes to the maternal lipid metabolism during pregnancy remains to be elucidated. In particular, the function of LOX-1 in placenta in context of preeclampsia remains largely unknown.

Recently several studies have highlighted the importance of heme oxygenase-1 (HO-1) in pregnancy (13, 14). HO-1 is an inducible enzyme that catalyzes the degradation of heme, yielding biliverdin, iron, and carbon monoxide, a potent vasodilator (15). It has been implicated in several physiological functions including the control of vascular tone (16) and the regulation of the inflammation (17) as well as contributing to the antioxidant capabilities (18). It has been reported that the protein level of HO-1 is decreased in preeclamptic placentas (17). Overexpression of HO-1 in endothelial cells results in a significant decrease in production of soluble fms-like tyrosine kinase 1 (sFlt-1) and soluble endoglin (14). They are the most important antiangiogenic circulating factors that are tightly associated with preeclampsia (19, 20). Furthermore, induction of HO-1 attenuates hypertension in pregnancy hypertension model animals (13). However, the primordial cause for the decrease of HO-1 in preeclamptic placenta has yet to be elucidated. Nuclear factor erythroid 2-related factor 2 (Nrf2) is a key molecule that is activated in response to oxidative stress and regulates antioxidant responsive element mediated induction of cytoprotective genes (21). Although Nrf2 is widely acknowledged as the predominant upstream regulator of HO-1 and is activated by oxLDL (22), the role of Nrf2 in placenta and how LOX-1 involves in Nrf2 activation have not been clarified.

In the present study, we hypothesized that LOX-1 may have an influence in preeclamptic placenta, leading to ma-

ternal pathogenesis of preeclampsia through high serum level oxLDL and endothelial dysfunction. We analyzed the expression and regulation of LOX-1 and postulated the relationship between LOX-1 and HO-1. We have also focused on Nrf2 and investigated its relevance to LOX-1 in preeclamptic placenta. Based on these results, we will discuss the putative role of LOX-1 and Nrf-2 on the pathogenesis of preeclampsia.

## Materials and Methods

### Patient characteristics and tissue collection

The clinical characteristics of patients enrolled in this study are shown in Table 1. Preeclampsia was defined as maternal systolic blood pressure of 140 mm Hg or greater and/or diastolic blood pressure of 90 mm Hg or greater in two consecutive measurements, with an interval of 6 h, and proteinuria of 300 mg or greater per 24 h after 20 wk of gestation. Placental villous tissues were collected from normal pregnant subjects ( $n = 16$ ) and preeclamptic subjects ( $n = 16$ ), immediately after cesarean section without labor at Kyoto University Hospital (Kyoto, Japan). The tissues were stored in RNAlater (Ambion, Austin, TX) after brief rinsing in saline. The local ethics committee of the Graduate School of Medicine, Kyoto University, approved the study protocol, and written informed consent was obtained from each patient enrolled in this study.

### Real-time quantitative RT-PCR

Total RNA extraction from placental tissues and cells was performed using the RNeasy Mini kit (QIAGEN, Germantown, MD) according to the manufacturer's instructions. RNA quality and quantity was measured using a ND-1000 spectrophotometer (Nanodrop, Wilmington, NC). Reverse transcriptsase of 1  $\mu$ g RNA was performed using the Rever Tra Ace (TOYOBO, Osaka, Japan) according to the manufacturer's instructions. The following primers were designed using GeneFisher 2 software (Bielefeld University Bioinformatics Service, Bielefeld, Germany): *LOX-1* (*OLR1*) (GenBank accession no. NM\_002543.3), 5'-CACCACCA GAATCTGAATCTCCAAG-3' (forward), 5'-TTCAGCAACTTG GCATCCAAAGAC-3' (reverse); *CD36* (NM\_001001548.2), 5'-TTGGAACAGAGGCTGACAACTTCAC-3' (forward), 5'-ATG GATCCCTATAGCCCCATAACAG-3' (reverse); *SR-A* (*MSR1*) (NM\_138716.2), 5'-CTCATTGGAATAGTGGCAGCTCAA C-3' (forward), 5'-CTTCTCCATGTTGCTCATGTGTTCC-3' (reverse); *sFlt-1* (U01134.1), 5'-AGGGGAAGAAATCCTCCA-3' (forward), 5'-CGAGCCTGAAAGTTAGCA-3' (reverse); leptin

**TABLE 1.** Clinical characteristics of the normal and preeclamptic (PE) patient groups

	Normal (n = 16)	PE (n = 16)	P value
Patient's age at delivery (yr)	35.6 $\pm$ 5.2 (27–44)	33.8 $\pm$ 4.0 (27–41)	n.s.
Primipara (n)	7/16	11/16	—
Gestational age at delivery (wk)	38 [37–38]	34 [32–37]	<0.01
Body mass index at delivery (kg/m <sup>2</sup> )	25.2 $\pm$ 2.1 (21.2–29.1)	25.0 $\pm$ 3.3 (21.0–33.4)	n.s.
Systolic blood pressure at delivery (mm Hg)	106 $\pm$ 6 (90–116)	168 $\pm$ 16 (145–192)	<0.0001
Diastolic blood pressure at delivery (mm Hg)	63 $\pm$ 8 (52–80)	101 $\pm$ 12 (80–124)	<0.0001
Neonatal weight (g)	2969 [2756.5–3189.5]	1583.5 [1290.5–2440]	<0.0001

Values are the mean  $\pm$  SD and (range) or median value with [interquartile range]. Dashes indicate the median value with interquartile range.

(*LEP*) (NM\_000230.2), 5'-GTGCCCATCCAAAAAGTCCA-AGAT G-3' (reverse); *HO-1* (*HMOX1*) (NM\_002133.2), 5'-CCAGGCAGAGAATGCTGAGTTCATG-3' (forward), 5'-TGCA GCTCTTCTGGGAAGTAGACAG-3' (reverse); glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (NM\_002046.3), 5'-GAGTCAACGGATTTGGTCGTATTGG-3' (forward), 5'-GCCA TGGGTGGAATCATATTGGAAC-3' (reverse). Real-time quantitative RT-PCR was performed with SYBR premix Ex TaqII (Takara Bio, Otsu, Japan) using the LightCycler 480 real-time PCR system (Roche Diagnostics, Mannheim, Germany) based on the following run conditions: 95 C for 30 sec for initial denaturing followed by 95 C for 5 sec and 60 C for 30 sec for 40 cycles. For dissociation after PCR amplification, the protocol included slow heating from 60 to 97 C to ensure amplification specificity. The end result for gene expression was estimated using comparative crossing point method for relative quantification. All data were normalized and expressed relative to the *GAPDH* gene as an internal control. All samples were run in duplicates and quantitative detection was averaged.

### Cytosolic and nuclear extract preparation

Placental tissues were homogenized in radioimmunoprecipitation assay buffer (50 mmol/liter Tris-HCl, pH 8.0; 150 mmol/liter sodium chloride; 0.5% sodium deoxycholate; 0.1% sodium dodecyl sulfate; 1.0% Nonidet P-40 substitute) supplemented with cocktail protease inhibitor Complete Mini (Roche Diagnostics). The homogenized tissues were centrifuged at  $10,000 \times g$  for 20 min 4 C, and the supernatant was saved as cytosolic extract from placental tissues. The cells were washed with ice-cold PBS, and cytosolic protein was extracted in the same manner. Nuclear proteins from tissues and cells were prepared using CellLytic NuCLEAR extraction kit (Sigma Aldrich, St. Louis, MO) according to the manufacturer's instructions. Protein concentrations were determined with a bicinchoninic assay protein assay kit (Thermo Scientific, Rockford, IL).

### Western blotting

Thirty micrograms of the cytosolic (for LOX-1 and HO-1 expression) or the nuclear (for Nrf2 expression) protein were separated on 10% (LOX-1), 12% (HO-1), or 7.5% (Nrf2) sodium dodecyl sulfate-polyacrylamide gels, respectively. The separated proteins were transferred onto nitrocellulose membranes, which were blocked with 5% fat-free milk overnight at 4 C. We confirmed an equal amount of protein loading by Ponceau S staining. The membranes were probed with rabbit polyclonal antibody against LOX-1 (1:250; ABGENT, San Diego, CA), rabbit monoclonal antibody against HO-1 (1:2000; Abcam, Cambridge, UK), or rabbit polyclonal antibody against Nrf2 (H-300) (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA). The blots were subsequently incubated with appropriate secondary antibody (1:10,000; Santa Cruz Biotechnology). Rabbit polyclonal antibody against  $\beta$ -actin (1:5000; Abcam) and mouse monoclonal antibody against TATA binding protein (1:3000; Abcam) were used for cytosolic and nuclear loading control, respectively. Signals were detected with ECL Plus Western blotting reagent (GE Healthcare, Buckinghamshire, UK) and visualized by ChemiDoc system (Bio-Rad Laboratories, Hercules, CA).

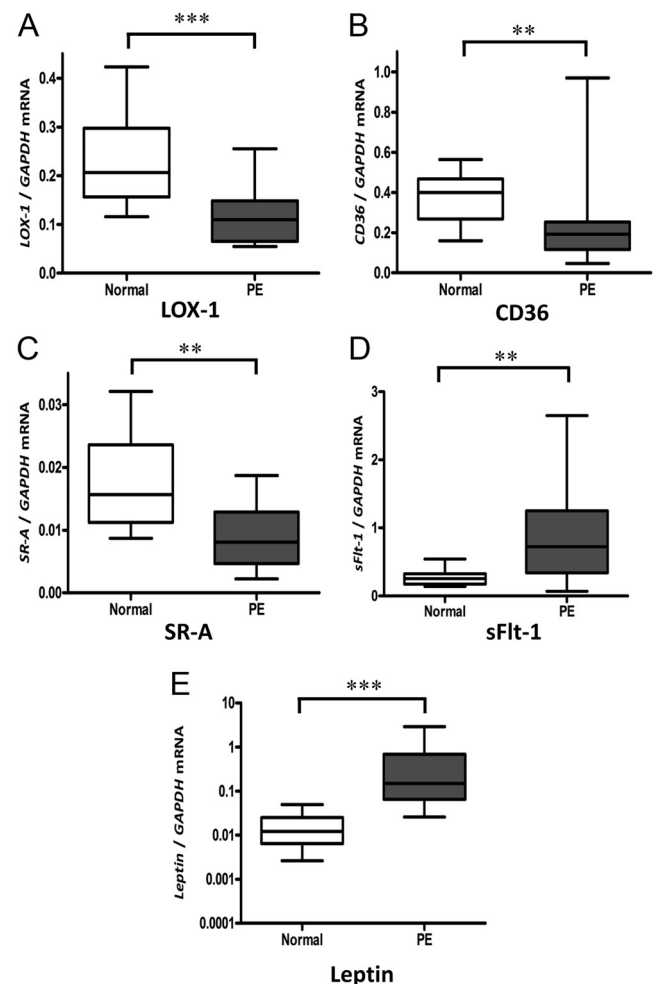
### Immunohistochemistry

Immunohistochemical staining was carried out using the streptavidin-biotin-peroxidase method. Formalin-fixed, paraf-

fin-embedded tissue sections were deparaffinized and antigen retrieval was performed in Tris-EDTA buffer (pH 9.0) at 120 C for 5 min. Endogenous peroxidase activity was blocked with 0.3%  $H_2O_2$ . The sections were incubated with rabbit polyclonal antibody against LOX-1 (1:50; ABGENT) or normal rabbit IgG overnight at 4 C, followed by incubation with biotinylated goat antirabbit secondary antibody (Nichirei, Tokyo, Japan). Then they were incubated with streptavidin-peroxidase complex solution for 30 min. Signals were generated by treatment with diaminobenzidine. Finally, the sections were counterstained with hematoxylin and observed under the microscope.

### Tissue and cell culture

Placental villous tissues were obtained from normal-term pregnancies delivered by elective cesarean section in the absence of labor. Small fragments of placental villi (~10–20 mg) were dissected from the placenta and washed in ice-cold PBS. Two fragments were placed in six-well plates with 3 ml culture medium (RPMI 1640 containing 10% fetal calf serum, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin) per well. For hypoxia treatment, explants ( $n = 6$ ) were cultured at 37 C, 5%  $CO_2$  in an atmosphere of 20 or 1%  $O_2$  for 24 h. The JAR (HTB-144) cho-



**FIG. 1.** Messenger RNA expressions in normal and preeclamptic placentas ( $n = 16$  in each group). *LOX-1* (A), *CD36* (B), *SR-A* (C), *sFlt-1* (D), *Leptin* (E) are shown. Values were normalized to those of *GAPDH*. Data are presented as the median value with interquartile range. \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .



riocarcinoma cell line was obtained from American Type Culture Collection (Manassas, VA) and cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin at 37 C, 20% O<sub>2</sub>, 5% CO<sub>2</sub>.

### Diethylmaleate (DEM) treatment

The JAR cells grown in six-well plates and a 10-cm dish for RNA extraction and protein extraction, respectively, were treated with or without 100  $\mu$ M DEM (Wako Pure Chemical Industries, Osaka, Japan), a typical Nrf2 activating electrophilic agent. After 3, 6, 9, and 24 h, Nrf2 protein and mRNA of HO-1 and LOX-1 were measured. The experiments were performed six times in duplicates ( $n = 6$ ). Placental explants ( $n = 6$ ) were

cultured at 37 C, 20% O<sub>2</sub>, 5% CO<sub>2</sub> with or without 100  $\mu$ M DEM. After 6 and 24 h, both protein and mRNA of HO-1 were measured.

### oxLDL treatment

oxLDL and native LDL (nLDL) were purchased from Intracel (Frederick, MD). Purified normal human IgG was purchased from R&D Systems (Minneapolis, MN). TS92, an antihuman LOX-1 antibody, was a kind gift from Dr. T. Sawamura (Osaka, Japan). JAR cells grown in 48-well plates or a 6-cm dish were treated with or without oxLDL (100  $\mu$ g/ml) for 3, 6, 9, and 24 h. The cells were also tested after being pretreated with TS92 (30  $\mu$ g/ml) or normal human IgG (30  $\mu$ g/ml) and later treated with oxLDL (100  $\mu$ g/ml) or nLDL (100  $\mu$ g/ml) for 9 h. After harvesting cells, mRNA and protein of HO-1 and Nrf2 was measured. The experiments were performed six times in triplicates ( $n = 6$ ).

### Statistical analysis

The results of normally distributed continuous variables are expressed as the mean  $\pm$  SEM (range), whereas those with skewed distribution were expressed as the median value with (interquartile range). Statistical comparisons were performed with a Mann-Whitney *U* test and a two-way ANOVA followed by Bonferroni test, and a one-way ANOVA followed by a Tukey test as appropriate, using Prism 4.0 (GraphPad Software, La Jolla, CA).  $P < 0.05$  was deemed statistically significant.

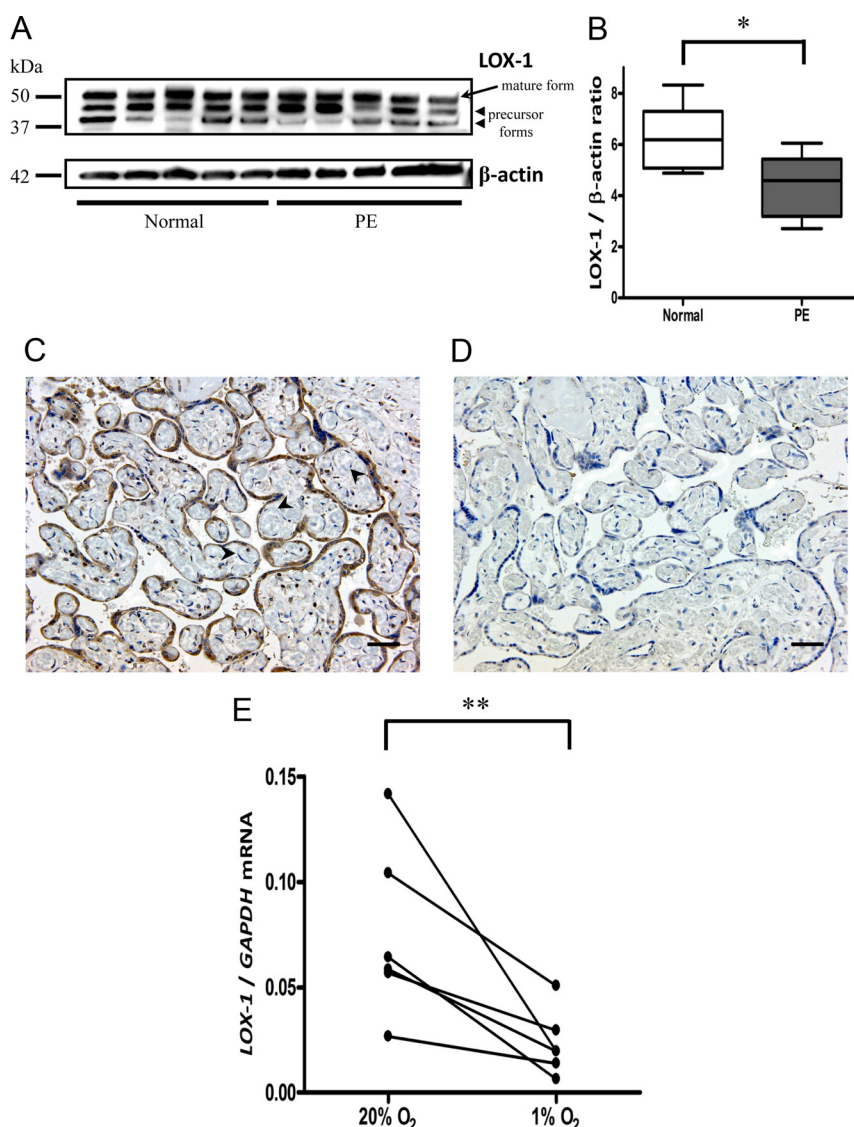
## Results

### Patient characteristics

The features of patients are shown in Table 1. Gestational age at delivery was earlier in the preeclampsia group than in the normal pregnancy group. Neonatal weight was lighter in the preeclampsia group than in the normal pregnancy group. Among 16 preeclamptic women, seven were early-onset ( $\leq 34$  wk gestation) preeclampsia and nine were late-onset ( $> 34$  wk gestation) preeclampsia. All patients are not habitual smokers and all preeclamptic women were diagnosed as severe preeclampsia.

### Expression of LOX-1 in normal and preeclamptic placentas

Quantitative real-time PCR analysis showed that mRNA expression of LOX-1 was significantly decreased in preeclamptic placentas compared with



**FIG. 2.** Expression of LOX-1 protein in placenta and mRNA expression under hypoxic condition in placental explant culture. A, Western blotting for LOX-1 in normal and preeclamptic placentas ( $n = 5$  in each group). An arrow indicates mature form of LOX-1 (52 kDa), and arrowheads show the precursor forms (40 kDa). B, The bands were quantified using densitometric analysis normalized to  $\beta$ -actin. The densitometric analysis was carried out on all bands. Data are presented as the median value with interquartile range. \*,  $P < 0.05$ . C, Immunohistochemical staining for LOX-1 in normal term placental villi. D, Negative control. Bar, 50  $\mu$ m. Arrowheads point at endothelial cells. E, LOX-1 mRNA expression in placental explants cultured in 20 or 1% O<sub>2</sub> ( $n = 6$ ). Values were normalized to those of *GAPDH*. \*\*,  $P < 0.01$ .

normal placentas ( $n = 16$  in each group) (Fig. 1A). The mRNA expressions of CD36 and SR-A, predominant receptors for oxLDL, were also significantly lower in preeclamptic placentas (Fig. 1, B and C). We confirmed the significantly higher mRNA of sFlt-1 and leptin in preeclamptic placentas compared with normal controls because they are well known to be up-regulated in preeclamptic placenta (Fig. 1, D and E). Western blot analysis of placental lysates demonstrated that protein level of LOX-1 was significantly reduced in preeclamptic placentas compared with the normal controls ( $n = 5$  in each group) (Fig. 2, A and B). With immunohistochemistry, LOX-1 immunostaining was observed in villous trophoblasts in normal term placenta; on the other hand, this was not detected in vascular endothelial cells (Fig. 2, C and D).

### The mRNA expression of LOX-1 in placental explants under hypoxic condition

We cultured placental explants under normal and hypoxic conditions (20 and 1%  $O_2$ , respectively) ( $n = 6$  in each group). After 24 h cultures, hypoxia resulted in significantly decreased LOX-1 mRNA expression (Fig. 2E).

### Nrf2 activation and HO-1 mRNA are decreased in preeclamptic placentas

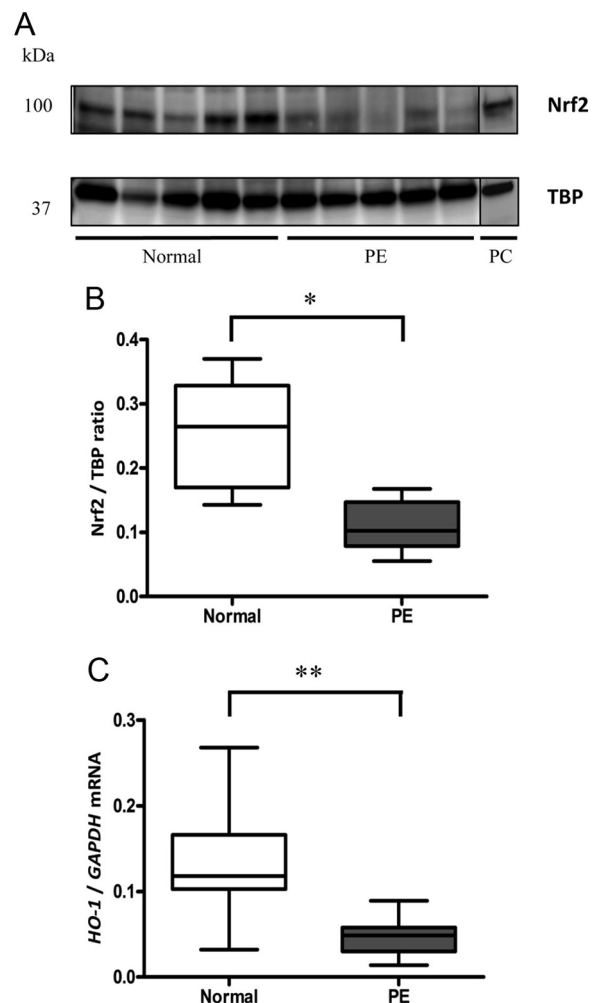
We analyzed Nrf2 activation by Western blot analysis using nuclear extracts from normal and preeclamptic placentas ( $n = 5$  in each group). Nuclear accumulation of Nrf2 was significantly decreased in preeclampsia compared with normal controls (Fig. 3, A and B). Next, we assessed mRNA expression of HO-1, which is a main target gene of Nrf2, and found significant low expression of that in preeclamptic placentas by quantitative real-time PCR ( $n = 16$  in each group) (Fig. 3C).

### Regulation of HO-1 mRNA by Nrf2 activation

We cultured the JAR choriocarcinoma cell line with or without DEM (100  $\mu M$ ), a typical Nrf2-activating agent. DEM administration significantly up-regulated HO-1 mRNA at 6 h and then decreased (Fig. 4A). The DEM treatment also augmented nuclear accumulation of Nrf2 and HO-1 protein expression after 6–9 h (Fig. 4B), whereas it did not up-regulate LOX-1 mRNA (Fig. 4C). In cultured placental explants, DEM (100  $\mu M$ ) significantly increased HO-1 mRNA at 6 h and then decreased (Fig. 4D). The HO-1 protein expression was also increased by DEM treatment (Fig. 4E). The experiments were performed six times in duplicates ( $n = 6$ ).

### Role of LOX-1 on the regulation of HO-1 by oxLDL

In JAR cells, HO-1 mRNA was up-regulated by oxLDL (100  $\mu g/ml$ ) and increased in a time-dependent manner up

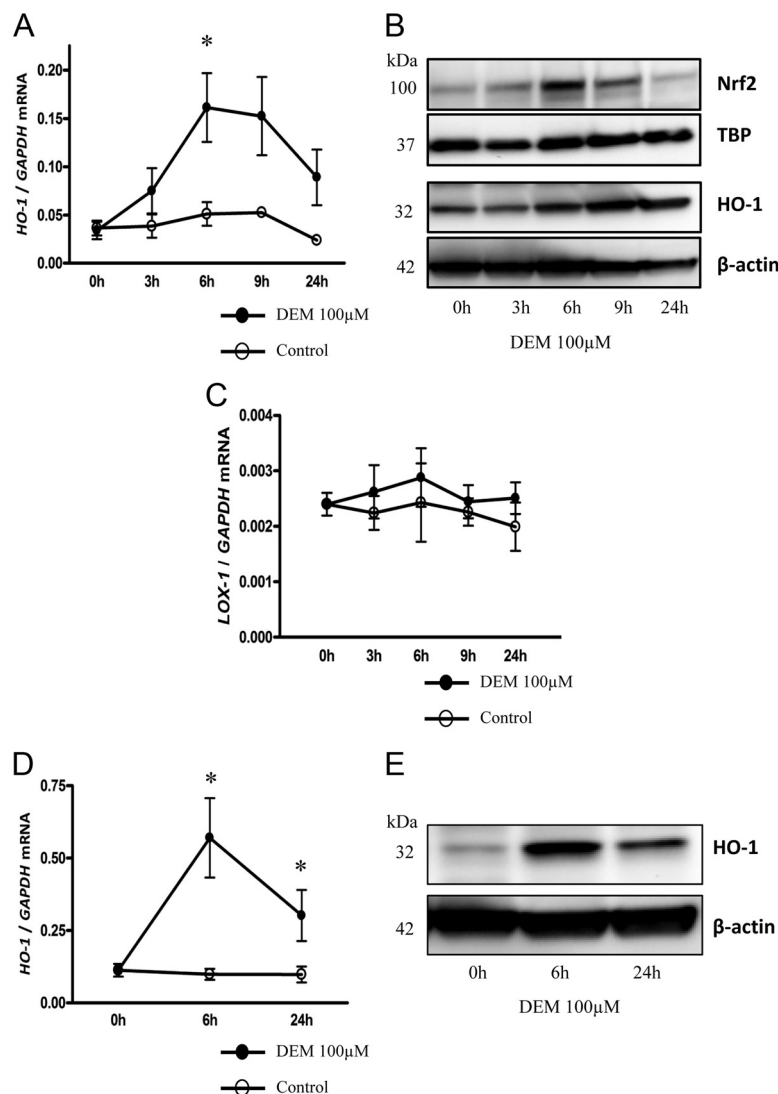


**FIG. 3.** Nrf2 activation ( $n = 5$  in each group) and HO-1 mRNA expression ( $n = 16$  in each group) in normal and preeclamptic (PE) placentas. A, Western blotting for Nrf2 using nuclear extract from normal and preeclamptic placentas. The far right lane contains nuclear extract of Hela cells treated with DEM (100  $\mu M$ ) as a positive control (PC). B, Densitometric analysis of Nrf2 expression normalized to TATA binding protein (TBP). C, HO-1 mRNA expression in normal and preeclamptic placentas. Values were normalized to those of GAPDH. Data are presented as the median value with interquartile range. \*  $P < 0.05$ , \*\*  $P < 0.01$ .

to 9 h and then decreased (Fig. 5A). Both nuclear accumulation of Nrf2 and HO-1 protein expression were increased by oxLDL treatment (Fig. 5B). In the same culture model, we inhibited the LOX-1 mediated signal by TS92, an antihuman LOX-1 antibody, and this pretreatment significantly alleviated the HO-1 up-regulation induced by oxLDL for 9 h, whereas nLDL did not affect HO-1 mRNA expression (Fig. 5C). The experiments were performed six times in duplicates ( $n = 6$ ).

### Discussion

In the present study, we first accurately demonstrated that LOX-1 expression in preeclamptic placentas is decreased



**FIG. 4.** Effect of DEM on JAR cells and placental explant culture. Time course of HO-1 (A) and LOX-1 (C) mRNA expression in JAR cells treated with DEM (100 μM). Values were normalized to those of GAPDH. B, A representative Western blotting image for the time courses of Nrf2 nuclear accumulation and HO-1 expression in cytosolic protein in JAR cells treated with DEM (100 μM). D, Time courses of HO-1 mRNA expressions in placental explant treated with DEM (100 μM). Values were normalized to those of GAPDH. E, A representative Western blotting image for the time course of HO-1 expression in cytosolic protein of placental explant treated with DEM (100 μM). Data are presented as the mean ± SEM. \*,  $P < 0.05$ . The experiments were performed six times in duplicates ( $n = 6$ ).

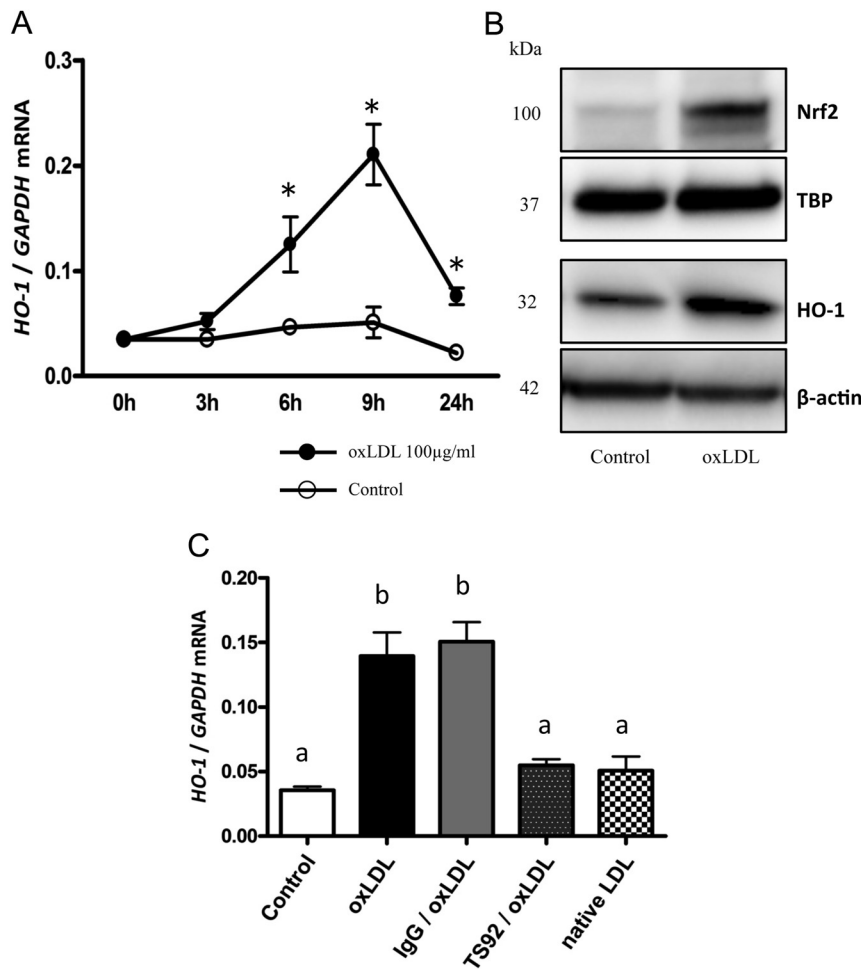
at both the mRNA and the protein level. Although LOX-1 is the most abundant in placenta, there have been few studies on LOX-1 in placenta. Contrasting to our results, Lee *et al.* (23) reported the elevated LOX-1 expression in the placentas of women with preeclampsia by Western blotting and immunohistochemistry. It is unclear what causes this discrepancy. The differences in phenotype of preeclamptic patients enrolled in these studies may be one of the reasons. We tried to collect placental tissues from the homogenous group with regard to mode of delivery, age, body mass index, and other factors. In addition, to confirm the quality of the samples, we also evaluated the

sFlt-1 and leptin, in which the up-regulation in preeclampsia are well established (19, 24), and we found both genes were significantly increased in our preeclamptic placentas. We therefore believed the samples were appropriately obtained from preeclamptic placentas and concluded that LOX-1 expression is significantly decreased in preeclamptic placentas. Ethier-Chiasson *et al.* (25) demonstrated that placental LOX-1 expression is higher in women with hyperlipidemia or gestational diabetes mellitus. Satoh *et al.* (26) have shown higher expression of LOX-1 mRNA in the first-trimester placenta than in the term placenta and suggested a connection with increasing oxidative stress at the end of the first trimester in placenta. It is reasonable that hyperlipidemia, gestational diabetes mellitus, or oxidative stress may cause the increase of LOX-1; however, our results suggested that LOX-1 is not increased in preeclamptic placenta despite oxidative stress, although the mechanism has not been elucidated.

In Western blot analysis, we detected three bands for LOX-1. Similar results were reported previously and suggested that two of the three bands were precursor forms, and the other one was the mature form (27). LOX-1 is synthesized as a precursor form and processed into mature form by glycosylation (28). Xie *et al.* (29) found that the extracellular C-terminal lectin-like domain is sufficient for the binding to oxLDL, and this domain is not glycosylated, which suggests the glycosylation of LOX-1 is not a prerequisite for the binding of ligand. Accordingly, we regarded these three signals as functional LOX-1 and quantified. Immunohistochemistry revealed that LOX-1 immunostaining was observed in villous trophoblasts but not in vascular endothelial cells. This is similar to the previous report by Satoh *et al.* (26), and LOX-1 is expressed mainly in trophoblasts.

We also demonstrated the significantly decreased LOX-1 mRNA in cultured placental explants under hypoxic condition, indicating that hypoxia can down-regulate LOX-1 expression in placenta. Although several stimuli or pathological conditions have been studied to





**FIG. 5.** Induction of HO-1 by oxLDL and effect of anti-LOX-1 antibody. **A**, Time courses of HO-1 mRNA expression in JAR cells treated with oxLDL (100 µg/ml). **B**, A representative Western blotting image of Nrf2 nuclear accumulation and HO-1 expression in cytosolic protein in JAR cells treated with oxLDL (100 µg/ml). **C**, HO-1 mRNA expression in JAR cells treated with oxLDL (100 µg/ml) in the presence or absence of TS92 (30 µg/ml) or normal human IgG (30 µg/ml) and treated with nLDL (100 µg/ml) for 9 h. Values were normalized to those of GAPDH. Data are presented as the mean ± SEM. \*,  $P < 0.05$ . Different letters denote significant difference ( $P < 0.001$ ). The experiments were performed six times in triplicates ( $n = 6$ ).

enhance LOX-1 gene expression (11, 12), little is known so far with regards to factors reducing LOX-1 expression. Here, for the first time, we revealed the pathological condition that down-regulates LOX-1 expression in placenta. Hypoxia is one of the factors involved in the onset of preeclampsia. At the early stage of pregnancy, impaired invasion and adaptation of extravillous trophoblasts to maternal spiral artery leads to reduced uteroplacental perfusion and placental hypoxia (2). It has been implicated in the pivotal pathogenesis of preeclampsia. So the decrease in LOX-1 expression may be derived from the early pathological alteration in placenta.

LOX-1 expression in placenta is the most abundant among the organs (9), and it can be the key molecule to regulate the serum oxLDL level. Ishigaki *et al.* (30) reported an actual example demonstrating the involvement

of LOX-1 in systemic lipid metabolism regulation. Hepatic LOX-1 overexpression enhanced oxLDL uptake in apolipoprotein E-deficient mice, and the plasma oxLDL level was markedly decreased. In women with preeclampsia, serum lipid levels are higher (4), and elevated oxLDL can be a risk factor of preeclampsia (31), suggesting that aberrant lipid metabolism may have a role in the pathogenesis of preeclampsia. In addition to LOX-1, CD36 and SR-A are considered predominant receptors for oxLDL (22), and mRNA expressions of both were revealed to be lower in preeclamptic placenta in the present study. These results suggest that the scavenger function against oxLDL is weakened in preeclamptic placentas. Therefore, it can be speculated that hypoxia at an early stage lead to decreased oxLDL receptors, and consequently, it may cause increasing serum oxLDL, which can give rise to further maternal endothelial dysfunction.

Next, we focused on Nrf2, a transcriptional factor that regulates antioxidant responsive element mediated induction of cytoprotective genes in response to oxidative stress (21). Under basal conditions, Nrf2 is sequestered in cytoplasm by binding to the Kelch-like ECH-associated protein 1 and is degraded by a proteasome pathway (32). However, upon exposure to oxidative or electrophilic stress, Nrf2 is dissociated from Kelch-like ECH-associated protein 1, accumulates in the nucleus, and induces the antioxidant genes.

Nrf2 is widely acknowledged as the predominant upstream regulator of HO-1. Despite its importance, there has been only one study that dealt with Nrf2 activation in placenta so far, providing nuclear accumulation of Nrf2 in cytotrophoblasts by immunohistochemistry (33). We showed here not only significantly lower Nrf2 activation but also decreased HO-1 mRNA in preeclamptic placentas. To investigate the role of Nrf2 in placenta, we examined the effect of DEM, an Nrf2 activator, in the JAR cell line and placental explant culture. We found that DEM certainly up-regulated HO-1 expression in both JAR cells and placental explants.

Although it is reported that oxLDL activates Nrf2 in murine macrophages (22), Nrf2 was less activated in pre-



eclamptic placenta despite a high serum level of oxLDL and increasing oxidative stress in women with preeclampsia. To address this query, we assessed the role of LOX-1 in JAR cells using TS92, a blocking anti-LOX-1 antibody. We confirmed in advance that only LOX-1, but neither CD36 nor SR-A, was expressed in JAR and considered that blockade of LOX-1 was enough to prevent JAR cells from oxLDL uptake. We revealed that oxLDL activated Nrf2 and up-regulated HO-1 expression in JAR cells and that LOX-1 blockade resulted in the alleviation of increasing HO-1 mRNA induced by oxLDL. These results suggest that oxLDL might be less internalized due to decreased LOX-1 in preeclamptic placenta than in the healthy state, leading to lower Nrf2 activation. When Nrf2 is not activated appropriately in trophoblasts, they fail to increase antioxidative genes, and both the mother and fetus may be affected against oxidative stress. In this study, LOX-1 mRNA was unchanged by the Nrf2 activator. It is currently unclear how the LOX-1 expression was decreased in preeclamptic placenta, and that is now under investigation.

In conclusion, to the best of our knowledge, this is the first study describing decreased LOX-1 expression and reduced Nrf2 activation in preeclamptic placenta. In addition, we have also shown the relevance between LOX-1 and Nrf2 through the assessment of HO-1 expression induced by oxLDL. The decrease in LOX-1 expression may contribute not only to maternal high serum oxLDL in woman with preeclampsia, although this needs further investigation, but also to lower Nrf2 activation in placenta. Our findings provided novel insights into the crucial role of LOX-1 and Nrf2 in placenta and paved the way for the precise comprehension of the pathogenesis of preeclampsia.

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## References

1. Sibai B, Dekker G, Kupferminc M 2005 Pre-eclampsia. *Lancet* 365:785–799
2. Steegers EA, von Dadelszen P, Duvekot JJ, Pijnenborg R 2010 Preeclampsia. *Lancet* 376:631–644
3. Herrera E, Amusquivar E, Lopez-Soldado I, Ortega H 2006 Maternal lipid metabolism and placental lipid transfer. *Horm Res* 65(Suppl 3):59–64
4. Potter JM, Nestel PJ 1979 The hyperlipidemia of pregnancy in normal and complicated pregnancies. *Am J Obstet Gynecol* 133:165–170
5. Branch DW, Mitchell MD, Miller E, Palinski W, Witztum JL 1994 Pre-eclampsia and serum antibodies to oxidized low-density lipoprotein. *Lancet* 343:645–646
6. Uzun H, Benian A, Madazli R, Topcuoglu MA, Aydin S, Albayrak M 2005 Circulating oxidized low-density lipoprotein and paraoxonase activity in preeclampsia. *Gynecol Obstet Invest* 60:195–200
7. Mitra S, Deshmukh A, Sachdeva R, Lu J, Mehta JL 2011 Oxidized low-density lipoprotein and atherosclerosis implications in antioxidant therapy. *Am J Med Sci* 342:135–142
8. Roberts JM, Redman CW 1993 Pre-eclampsia: more than pregnancy-induced hypertension. *Lancet* 341:1447–1451
9. Sawamura T, Kume N, Aoyama T, Moriwaki H, Hoshikawa H, Aiba Y, Tanaka T, Miwa S, Katsura Y, Kita T, Masaki T 1997 An endothelial receptor for oxidized low-density lipoprotein. *Nature* 386:73–77
10. Mehta JL, Chen J, Hermonat PL, Romeo F, Novelli G 2006 Lectin-like, oxidized low-density lipoprotein receptor-1 (LOX-1): a critical player in the development of atherosclerosis and related disorders. *Cardiovasc Res* 69:36–45
11. Chen M, Nagase M, Fujita T, Narumiya S, Masaki T, Sawamura T 2001 Diabetes enhances lectin-like oxidized LDL receptor-1 (LOX-1) expression in the vascular endothelium: possible role of LOX-1 ligand and AGE. *Biochem Biophys Res Commun* 287:962–968
12. Chen M, Kakutani M, Minami M, Kataoka H, Kume N, Narumiya S, Kita T, Masaki T, Sawamura T 2000 Increased expression of lectin-like oxidized low density lipoprotein receptor-1 in initial atherosclerotic lesions of Watanabe heritable hyperlipidemic rabbits. *Arterioscl Thromb Vasc Biol* 20:1107–1115
13. George EM, Cockrell K, Aranay M, Csongradi E, Stec DE, Granger JP 2011 Induction of heme oxygenase-1 attenuates placental ischemia-induced hypertension. *Hypertension* 57:941–948
14. Cudmore M, Ahmad S, Al-Ani B, Fujisawa T, Coxall H, Chudasama K, Devey LR, Wigmore SJ, Abbas A, Hewett PW, Ahmed A 2007 Negative regulation of soluble Flt-1 and soluble endoglin release by heme oxygenase-1. *Circulation* 115:1789–1797
15. Kikuchi G, Yoshida T, Noguchi M 2005 Heme oxygenase and heme degradation. *Biochem Biophys Res Commun* 338:558–567
16. Kozma F, Johnson RA, Zhang F, Yu C, Tong X, Nasjletti A 1999 Contribution of endogenous carbon monoxide to regulation of diameter in resistance vessels. *Am J Physiol* 276:R1087–R1094
17. Ahmed A, Rahman M, Zhang X, Acevedo CH, Nijjar S, Rushton I, Bussolati B, St. John J 2000 Induction of placental heme oxygenase-1 is protective against TNF $\alpha$ -induced cytotoxicity and promotes vessel relaxation. *Mol Med* 6:391–409
18. Siow RC, Sato H, Mann GE 1999 Heme oxygenase-carbon monoxide signalling pathway in atherosclerosis: anti-atherogenic actions of bilirubin and carbon monoxide? *Cardiovasc Res* 41:385–394
19. Maynard SE, Min JY, Merchan J, Lim KH, Li J, Mondal S, Libermann TA, Morgan JP, Sellke FW, Stillman IE, Epstein FH,

- Sukhatme VP, Karumanchi SA 2003 Excess placental soluble fms-like tyrosine kinase 1 (sFlt1) may contribute to endothelial dysfunction, hypertension, and proteinuria in preeclampsia. *J Clin Invest* 111:649–658
20. Levine RJ, Lam C, Qian C, Yu KF, Maynard SE, Sachs BP, Sibai BM, Epstein FH, Romero R, Thadhani R, Karumanchi SA 2006 Soluble endoglin and other circulating antiangiogenic factors in preeclampsia. *N Engl J Med* 355:992–1005
  21. Itoh K, Chiba T, Takahashi S, Ishii T, Igarashi K, Katoh Y, Oyake T, Hayashi N, Satoh K, Hatayama I, Yamamoto M, Nabeshima Y 1997 An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. *Biochem Biophys Res Commun* 236:313–322
  22. Ishii T, Itoh K, Ruiz E, Leake DS, Unoki H, Yamamoto M, Mann GE 2004 Role of Nrf2 in the regulation of CD36 and stress protein expression in murine macrophages: activation by oxidatively modified LDL and 4-hydroxynonenal. *Circ Res* 94:609–616
  23. Lee H, Park H, Kim YJ, Kim HJ, Ahn YM, Park B, Park JH, Lee BE 2005 Expression of lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) in human preeclamptic placenta: possible implications in the process of trophoblast apoptosis. *Placenta* 26:226–233
  24. Poston L 2002 Leptin and preeclampsia. *Semin Reprod Med* 20:131–138
  25. Ethier-Chiasson M, Forest JC, Giguère Y, Masse A, Marseille-Tremblay C, Lévy E, Lafond J 2008 Modulation of placental protein expression of OLR1: implication in pregnancy-related disorders or pathologies. *Reproduction* 136:491–502
  26. Satoh H, Kiyota E, Terasaki Y, Sawamura T, Takagi K, Mizuta H, Takeya M 2008 Expression and localization of lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) in murine and human placentas. *J Histochem Cytochem* 56:773–784
  27. Serke H, Bausenwein J, Hirrlinger J, Nowicki M, Vilser C, Jogschies P, Hmeidan FA, Blumenauer V, Spanel-Borowski K 2010 Granulosa cell subtypes vary in response to oxidized low-density lipoprotein as regards specific lipoprotein receptors and antioxidant enzyme activity. *J Clin Endocrinol Metab* 95:3480–3490
  28. Kataoka H, Kume N, Miyamoto S, Minami M, Murase T, Sawamura T, Masaki T, Hashimoto N, Kita T 2000 Biosynthesis and post-translational processing of lectin-like oxidized low density lipoprotein receptor-1 (LOX-1). N-linked glycosylation affects cell-surface expression and ligand binding. *J Biol Chem* 275:6573–6579
  29. Xie Q, Matsunaga S, Shi X, Ogawa S, Niimi S, Wen Z, Tokuyasu K, Machida S 2003 Refolding and characterization of the functional ligand-binding domain of human lectin-like oxidized LDL receptor. *Protein Expr Purif* 32:68–74
  30. Ishigaki Y, Katagiri H, Gao J, Yamada T, Imai J, Uno K, Hasegawa Y, Kaneko K, Ogihara T, Ishihara H, Sato Y, Takikawa K, Nishimichi N, Matsuda H, Sawamura T, Oka Y 2008 Impact of plasma oxidized low-density lipoprotein removal on atherosclerosis. *Circulation* 118:75–83
  31. Sanchez SE, Williams MA, Muy-Rivera M, Qiu C, Vadachkoria S, Bazul V 2005 A case-control study of oxidized low density lipoproteins and preeclampsia risk. *Gynecol Endocrinol* 21:193–199
  32. Itoh K, Wakabayashi N, Katoh Y, Ishii T, Igarashi K, Engel JD, Yamamoto M 1999 Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. *Genes Dev* 13:76–86
  33. Wruck CJ, Huppertz B, Bose P, Brandenburg LO, Pufe T, Kadyrov M 2009 Role of a fetal defence mechanism against oxidative stress in the aetiology of preeclampsia. *Histopathology* 55:102–106



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